Diagnosis of the Hemolytic Anemias

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SUMMARY

Classification of the varieties of hemolytic anemia is based upon whether hemolysis is due to inherent defects within the erythrocyte or to extracellular factors.

Confirmation of diagnosis in cases in which the disease is clinically suspected is made upon the basis of the following laboratory data: Hyperbilirubinemia giving an indirect van den Bergh reaction, observation of typical factors in a smear of the blood, a reactive bone marrow, increased urobilinogen in stools and urine, and absence of bilirubinuria.

Differentiation of the different types depends upon determination of altered fragility of erythrocytes as indicated by tests of resistance to heat, acid, and mechanical and osmotic stimuli, and upon the identification of an antibody.

The importance of detecting antibodies demonstrable in acid serum is mentioned.

In a majority of cases, acquired hemolytic anemia is caused by antibodies, malignant disease, or toxic agents.

HEMOLYTIC anemia results from an increase above normal in erythrocyte destruction and beyond the compensatory ability of the bone marrow to match.

The average life-span of the normal erythrocyte is about 120 days. This has been conclusively established both in this country and abroad by transfusion experiments involving the introduction into normal persons of normal erythrocytes either tagged with P_{32} or of a different blood group than the blood of the recipient. These transfused erythrocytes may be identified by the Geiger counter or by differential agglutination. There is normal daily destruction and production of 7 to 8 gm. of hemoglobin and about 1 per cent of the circulating erythrocytes. Small increases in erythrocyte destruction may be equalized by increased marrow erythropoiesis, but when destruction outstrips production, anemia results.

The recognition of hemolytic anemia is important because anemia of this order does not respond to iron compounds, liver extract, folic acid, B_{12}, other vitamins, or other hematinics which may be given before the type of anemia is recognized. An accurate diagnosis should always be attempted in order to prognosticate the course of the illness, to avoid useless medication, and also for the very pragmatic therapeutic reason that in a few of the various kinds of hemolytic anemia dramatic results may be achieved by splenectomy or vigorous treatment of the underlying cause. Increased hemolysis may be caused by such diverse mechanisms as splenic destruction of spherocytes, perverse antibody production, leukemia and lymphoma, animal toxins, and chemical idiosyncrasy, to mention a few. Before classifying the hemolytic anemias, the recognition of abnormal hemolysis itself is necessary.

The purpose of this presentation is to state general principles and outline procedures which may be done in the average clinical laboratory. Complete techniques are given in detail by Ham[12, 13] in two recent publications.

In a carefully taken history and physical examination, anemia, icterus, splenomegaly, hepatomegaly, lymphadenopathy, leg ulcers, Raynaud-like phenomena, familial or past history of jaundice, contact with chemicals, or disease not infrequently associated with increased hemolysis will be noted. Clinical laboratory data are usually necessary to confirm the clinical impression and to differentiate between the different groups of hemolytic anemia.

RECOGNITION OF INCREASED HEMOLYSIS

Pigment reactions. Hemoglobin is broken down by the cells of the reticuloendothelial system to the pigment bilirubinglobin. This pigment does not pass the renal barrier, so it does not appear in the urine. It gives the indirect or delayed van den Bergh reaction. The parenchymal cells of the liver split off the globin molecule and excrete the resulting bilirubinate as the sodium salt into the bile, by which medium it reaches the intestines. The bacterial flora of the bowel reduce the bilirubin to the colorless chromogen urobilinogen, part of which is excreted in the feces, the rest being absorbed into the portal circulation. The greater portion is reexcreted in the bile or metabolized by the liver; small amounts are excreted in the urine. An increase in hemoglobin catabolism, therefore, results in an increase in serum bilirubinglobin, fecal bilirubin, and fecal and urinary urobilinogen.[22, 23]

The relative levels of serum bilirubinglobin and fecal urobilinogen are dependent upon the functional capacity of the liver. For example, in moderate hemolytic anemia with good liver function, there may be little or no increase in the icteric index or serum bilirubin, yet the fecal and urinary urobilinogen may be increased to several times normal. On the other hand, a person with a milder hemolytic

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anemia but with impaired liver function may have elevated serum bilirubin. The serum bilirubin alone is therefore not a reliable index of the degree of hemolysis.

Obstructive or hepatocellular jaundice produces increased serum bilirubin, which, unlike bilirubinglobin, appears in the urine and gives a positive direct or immediate van den Bergh reaction. Both bilirubin and bilirubinglobin give a similar icteric index and total bilirubin determination. Demonstration of the absence of bile in the urine will usually differentiate hemolytic from the other types of jaundice. Of the many tests, the best is Harrison's test[9] which employs the precipitation of the bilirubin by barium chloride and its subsequent oxidation to biliverdin by Fouchet's reagent.

The absolute proof of increased hemolysis is the demonstration of increased fecal urobilinogen. To be accurate, the determination should be done on at least a 24-hour specimen or preferably upon the fecal output of four days, the principal method being that of Watson.[24] An increase above 250 mg. in 24 hours is significant. Since not only the rate of hemoglobin destruction but the total mass of hemoglobin is important in determining the excretion of urobilinogen, Miller and Dameshek[14] calculate their hemolytic index as follows:

$$\frac{\text{24-hour fecal urobilinogen in mg. x 100}}{\text{Hemoglobin in gm. per 100 cc. x blood volume} \div 100}$$

The normal index is 11 to 20.

Practically speaking, the determination of 24-hour fecal urobilinogen is not done in most laboratories and the determination upon a single fecal specimen may be misleading. The determination of urobilinogen in a fresh afternoon urine specimen will give a working approximation of the fecal urobilinogen. The method of Wallace and Diamond,[21] which employs the addition of Ehrlich's aldehyde reagent to twofold dilutions of urine, is simple. If a pink color is detected in a dilution of greater than 1:20, the urobilinogen is increased. False low values may obtain if the urine is allowed to stand so that the urobilinogen is oxidized to urobilin or if the oral administration of antibiotics has changed the intestinal flora so that the bilirubin is not reduced to urobilinogen.[10] In porphyria, porphobilinogen will give a positive reaction in high dilution.

Intravascular hemolysis will increase the plasma hemoglobin above the normal 5 mg. per 100 cc. Gross increases may be detected by the pink color of the plasma. Bing and Baker[1] have developed a quantitative benzidine method. It is necessary, of course, to use extreme care in drawing the blood in order not to produce technical hemolysis in the test tube.

The urine should always be tested for hemosiderin. A simple test in which the urine sediment is mixed with an equal part of 30 per cent ammonium sulfide solution has been devised by Cook.[7] The reaction to this test is always positive in nocturnal hemoglobinurina, and frequently in other hemoglobinurias.

Hematological reactions. The erythrocytes vary from normocytic to macrocytic, the degree of macrocytosis being dependent somewhat upon the rapidity of erythrocyte regeneration. Except in Cooley's anemia and uncommonly in sickle-cell anemia in the young, the cells are well filled with hemoglobin. Polychromatophilia and nucleated erythrocytes are roughly proportional to the severity of the anemia and the regenerative ability of the bone marrow. Frequently there is basophilic stippling of the erythrocytes. With severe hemolysis, especially if it occurs intravascularly, fragmented and ghost cells may be observed on the blood smear. In Cooley's anemia and sickle-cell anemia, target cells are present.

Supravital staining with cresyl blue permits counting of the reticulocytes, the best single measure of the physiological activity of the bone marrow. During the regenerative phase following a hemolytic crisis the reticulocytes may number 80 to 90 per cent of the total erythrocytes. A normal reticulocyte count is rare in hemolytic anemia, although Dameshek[7] recently gave examples of acquired hemolytic anemias with low reticulocyte counts before therapy with ACTH. After therapy there was reticulocytosis. (The author has seen one example of this.)

Spherocytes are readily identified on the stained smear by the technician who looks for them. They are to be seen on those well spread portions of the smear where the erythrocytes are neither crenated by the slow drying or excessively spread out. A decreased mean cell diameter with normal mean corpuscular volume and normal mean corpuscular hemoglobin indicates spherocytosis. On a wet mount, spherocytes may be recognized by their rounded shape and inability to form long rouleaux. Spherocytes indicate hemolytic anemia—unless the patient has recently received a blood transfusion. Transfused cells, especially if they have been previously stored, may become spherical. A spherocyte is a congenitally fragile cell or one which has become damaged by some means.

The blood platelets are usually normal unless altered by the underlying cause of the hemolysis.

Unless influenced by the primary condition, the number of leukocytes is normal or often increased with a relative increase in the number of immature neutrophils during acute hemolysis. Evans[8] noted that in certain hemolytic anemias there is thrombocytopenia and neutropenia, suggesting the relationship of these three entities and abnormal antibody formation. Pronounced erythroid hyperplasia occurs in the bone marrow, involving all stages of erythrocyte maturation but especially the pronormoblast phase. A reversed myeloid-erythroid ratio with many pronormoblasts and orthochromatic normoblasts suggests the possibility of hemolytic anemia but bone marrow examination is chiefly of value in ruling out megaloblastic anemia, hypoblastic anemia, and leukemia.

The previously mentioned basic tests on blood, bone marrow, and urine may be done in any clinical laboratory or in the physician's office and the

results will usually establish the presence or absence of hemolytic anemia. The disease may be any of the various kinds in the following classification, and there are means for the specific diagnosis of each kind:

CLASSIFICATION OF THE HEMOLYTIC ANEMIAS

Anemias due to defect in the erythrocyte

1. Congenital hemolytic anemia
2. Sickle-cell anemia
3. Cooley's anemia
4. Congenital nonspherocytic hemolytic anemia (Haden)
5. Nocturnal hemoglobinuria
6. Ovalocytosis with hemolytic anemia
7. Pernicious anemia (the role of hemolysis is a definite although a minor one)

Acquired hemolytic anemias due to causes other than erythrocyte defect

1. Protozoan parasites; malaria
2. Bacterial or animal toxins: *B. Welchii*, snake venom
3. Chemical agents: Chlorates, benzene
4. Thermal burns
5. Intravascular hypotonic solutions: Prostatic resection
6. Symptomatic group: leukemias and lymphomas.
7. Allergic sensitization: Favism
8. Antibody group
   a) Iso-agglutinin reactions: Incompatible blood transfusions, erythroblastosis fetalis
   b) Paroxysmal cold hemoglobinuria (syphilitic)
   c) Anemia due to cold hemagglutinins
   d) Anemia due to "irregular" iso- and auto-hemagglutinins. Most of these were formerly classified as idiopathic acute or chronic hemolytic anemia
9. March hemoglobinuria

Rather than attempt to run through the differential points in the diagnosis of these anemias, special tests of hemolysis and those anemias diagnosed by the tests will be discussed. The order in which the tests should be done is dependent upon the judgment of the clinician. For example, for a young Negro with anemia, the sickle-cell test should be done first. For an older person with a history of red urine and a positive reaction to a Kahn test of the blood, only the Donath-Landsteiner test may be required to establish the diagnosis of paroxysmal cold hemoglobinuria due to syphilis.

SPECIAL DIAGNOSTIC PROCEDURES

Osmotic fragility. An increase in osmotic fragility is present constantly in congenital hemolytic anemia and inconstantly in various kinds of acquired hemolytic anemia. In sickle-cell anemia and Cooley's anemia decreased osmotic fragility occurs. A screen test is done by adding 0.1 ml. of blood to two test-tubes containing 1 ml. of 0.85 per cent and 0.50 per cent of sodium chloride solution respectively.[12] If hemolysis occurs in the 0.50 per cent solution, then further test should be done by Creed's technique[4] which utilizes a series of tubes with saline solution varying from 0.28 per cent to 0.72 per cent with intervals of 0.04 per cent or the more modern quantitative technique utilizing the photoelectric-cell colorimeter.[20]

Detection of antibodies. The antibody-antigen nature of many of the acquired hemolytic anemias was suspected for years. It was impossible, however, using the conventional method of titrating saline erythrocyte suspensions against the suspected serum, to demonstrate agglutination, except in a few instances.

Rh-positive erythrocytes which would be expected to be agglutinated by the serum from sensitized mothers sometimes did not agglutinate. Indeed, the maternal serum from mothers of erythroblastotic children might inhibit the agglutination of Rh-positive cells by serum known to contain saline effective agglutinins. Thus was developed the blocking antibody test which indicated that more than one type of antibody may be responsible for erythroblastosis fetalis.[25]

Wiener, followed by a host of other investigators, showed that substitution of 20 per cent albumin solution (or many other colloidal media) for saline solution would permit the demonstration of antibodies not only in the serum of mothers with erythroblastotic infants but also in the serum of patients with many kinds of acquired hemolytic anemia which had previously been called idiopathic. Thus was born the conglutination test which, in its modifications, is extensively used.[16, 26] The blocking antibody test is little used at present.

Coombs[3] found that the antibodies absorbed on the erythrocytes of babies with erythroblastosis fetalis could not be removed by repeated washing with normal saline solution. He found that serum from rabbits which had been immunized against human globulin would agglutinate these sensitized cells. This is Coombs' test. The next step was the demonstration of a positive reaction to Coombs' test in certain acquired hemolytic anemias.[11] The direct Coombs' test detects only antibodies absorbed on the cell. To detect antibodies in serum, an indirect Coombs' test is employed. Normal red cells of the same group or group O and varying Rh types are incubated with the serum in question and then, after they have been washed, are exposed to Coombs' serum. A quantitative titration of antibodies may be done by the indirect Coombs' technique, thus supplementing the conglutination test.[12]

The Coombs' test is not invariably negative in congenital hemolytic anemia, as was first thought.

An indirect Coombs' titration will generally detect antibodies in higher titre than the conglutination test. Either or both the direct and indirect Coombs' tests may demonstrate antibodies not detectable by other techniques.

Erythrocytes exposed to a solution of trypsin become more readily agglutinable by serum containing antibodies against this particular cell group. Trypsinized cells Rh_1Rh_2 (CDE-CDE) may be used to detect sensitivity to the Rh group antigens. This will provide an extra safeguard for prospective recipients of blood transfusions.[15]

Persons with acquired hemolytic anemia may possess hemagglutinins or hemolysins effective only in an acid medium so that results of a conglutination or indirect Coombs' test may be negative if done in unaltered serum. The acidification of the serum from the patient will detect these antibodies.[15]

Persons with viral pneumonia or with no other disease at all, may develop hemagglutinins which produce erythrocyte agglutination in the cold. The condition should be looked for as the cause in obscure anemia. An alert technician may provide data by which to establish the diagnosis by observing the clumping of the erythrocytes in the counting chamber. These antibodies do not require complement for agglutination. Mechanical agitation of the clumped cells produces hemolysis; this is probably the mechanism in the body where exposure to cold produces intravascular hemolysis and hematuria. Hemosiderin in the urine is commonly found. With high titres, the erythrocytes may clump at a temperature close to that of the body; with low titres, exposure to 4° C. may be necessary. The reaction to the Donath-Landsteiner test is negative.[18, 27]

Syphilitic paroxysmal cold hemoglobinuria is caused by a hemolysin which becomes fixed to the erythrocytes at 4° C. and produces hemolysis when the erythrocytes are then warmed to 37° in the presence of complement. The defect is not in the erythrocyte; the hemolysin will hemolyze normal cells of Group O or of the same blood group as that of the patient. The sensitized but unhemolyzed cells will give a positive reaction to a direct Coombs' test.[12] A person with positive reaction to a serologic test for syphilis who passes red urine after immersion of a limb in ice water may be presumed to have paroxysmal cold hemoglobinuria. Confirmation can be made by the Donath-Landsteiner test which is not difficult to carry out. Caution should be exercised in chilling a patient suspected of having this disease, as excessive chilling may produce a severe systemic reaction with chills, fever, shock, and urticaria as well as the blood in the urine. A person with hemolytic anemia due to cold hemagglutinins, which were previously discussed, may also have blood in the urine after chilling.

Hemolysis at acid pH (Ham's test).[12] In nocturnal hemoglobinuria the erythrocytes have a defect which causes them to be hemolyzed by the patient's or normal serum at an acid pH. The factor present in normal serum which is necessary for hemolysis is heat-labile but is not complement; it may be Ac-globulin.[5] The complete test is time-consuming, as it involves the following 16 combinations:

5 Per Cent Suspension of Erythrocytes from:	Serum		Acidified Serum		Inactivated Serum		Inactivated Serum Plus Complement	
	P*	C*	P	C	P	C	P	C
Patient	±	±	+	+	0	0	0	0
Normal control	0	0	†	0	0	0	0	0

*P=patient; C=control.

†Certain cases of hemolysin or hemagglutinin effective at acid pH will produce hemolysis in this tube.

The complete test is rather time-consuming and if the pH is too low, hemolysis of normal cells may occur. Thus a simple screening test suggested by Ham[12] is useful. Five milliliters of clotted blood from the patient and the same amount from a control are incubated at 37° C. and observed for six and 12 hours. Hemolysis in the tube containing the blood from the patient suggests nocturnal hemoglobinuria, or acquired hemolytic anemia due to an antibody, or sometimes congenital hemolytic anemia; and if that occurs, the complete Ham test must be done.

Detection of sickling. For a long time it has been known that in about 7 per cent of the American Negroes some of the erythrocytes assume a sickle shape when the hemoglobin is in a reduced form. This property is hereditary, being dominant. Pauling[17] observed that the hemoglobin in cells that sickle is of a different molecular weight than normal hemoglobin. The majority of persons in whom this phenomenon occurs have no symptoms but 1 per cent of them have hemolytic anemia with all the characteristics of increased hemolysis. In examination of blood from persons with severe forms of the disease, sickling may be observed on the blood smear or in the counting chamber. This obvious sickling associated with reticulocytosis, leukocytosis, nucleated erythrocytes, and target cells leaves no doubt as to the diagnosis. In the sickle-cell trait and in mild forms of the disease, however, special techniques are required to bring out sickling.

A simple technique has been used for this purpose at the Los Angeles County Hospital. A drop of blood is placed upon a drop of dried cresyl blue on a slide, a cover slip placed over the drop and the edges sealed with vaseline. The preparation is examined at the end of 24 hours for sickling, and at the same time a reticulocyte count is done. Sickling may be demonstrated within 30 minutes by equilibrating the blood with carbon dioxide or by using reducing agents such as sodium metabisulfite.[6]

Mechanical fragility. The measurement of mechanical fragility is not adaptable to the ordinary laboratory. At present the information gained does not warrant its use except in research. The technique is given by Ham.[13]

Miscellaneous conditions. Diagnosis of hemolytic anemia of the symptomatic group depends upon demonstration of an underlying disease known to produce hemolysis. Inconstant spherocytosis, increased osmotic, acid, and mechanical fragility, and a positive reaction to direct Coombs' test[19] may be observed. Hemagglutinins may be inconstantly demonstrated in the serum by the indirect Coombs' test or conglutination test.

There is at the present no practical laboratory test for the confirmation of the diagnosis of Cooley's anemia. A Mediterranean parentage and a blood smear characteristic of thalassemia minor in at least one of the parents will establish the diagnosis.

Hypochromic anemia that is not due to chronic blood loss or infection and which does not respond

to adequate doses of parenteral iron may be Cooley's anemia.

Toxic hemolytic anemia due to chemicals is generally acute. Diagnosis depends upon the exclusion of the other causes of hemolysis and a history of prolonged or extensive exposure to chemicals known to be capable of producing hemolytic anemia. Abrupt onset in persons previously healthy is an important clue. Generally antibodies are not demonstrable.

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